high ROS levels (Benani et al., 2007; Andrews et al., 2008; Horvath et al., 2009; Jaillard et al., 2009). Repeated activation of POMC neurons and related behaviors and autonomic adaptations occur daily. Short-term ROS peaks appear to be fundamental for evoking a proper behavioral, endocrine, and autonomic response to nutrient intake and are likely to be associated with short-term peaks of ROS generation in sympathetic neurons as well. On the other hand, prolonged exposure to hyperglycemia-triggered ROS clearly impairs sympathetic neuronal functions and outflow due to oxidation of the Cys residue of cholinergic receptors (Campanucci et al., 2010). Whether similar impairments of receptor activation (not necessarily cholinergic receptors) occur in POMC neurons in response to sustained ROS generation, when an animal is on high fat diet for example, is a highly relevant question to pursue.

In summary, the observations of Campanucci et al. (2010) shed new light on the etiology and offer potential new therapeutic approaches for diabetic neuropathies. Questions remain, however, regarding the source of ROS that impair nAchRs function: whether ROS are from intracellular or extracellular origin and whether ROS are the product of glucose oxidation or emerge from other metabolic processes. Further work is also needed to clarify whether effects of neuropathies promoted by diabetes influence hepatic glucose production and output and how restoration of sympathetic outflow would impact these critical processes.

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Alternative Splicing Disabled by Nova2

Tae-Ju Park[1] and Tom Curran[1,*]
[1]Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
*Correspondence: currant@email.chop.edu
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Disabled-1 is a key signaling molecule in the Reelin pathway that plays a critical role in neuronal migration and positioning during brain development. In this issue of *Neuron*, Yano et al. demonstrate that the neuron-specific RNA binding protein Nova2 contributes to neuronal migration by regulating alternative splicing of disabled-1.

Neuronal migration and positioning in the developing brain is a complicated process that requires well-orchestrated interactions among migrating neurons, radial glial cells, and postmigration neurons. The Reelin pathway controls neuronal migration in the developing cortex, cerebellum, and hippocampus, and it has been implicated in human brain disorders such as lissencephaly, schizophrenia, bipolar disorder, autism, and temporal lobe epilepsy. Genetic and biochemical studies have helped elucidate the biochemical mechanisms responsible for Reelin signaling (for review, see Rice and Curran 2001; Ayala et al., 2007). Reelin is a protein ligand that binds two receptors: apoER2 and VLDLR. Binding of Reelin to these receptors triggers tyrosine phosphorylation of disabled-1 (Dab1) by Fyn and Src. Tyrosine phosphorylated Dab1 recruits downstream signaling molecules including the SH2/SH3 domain-containing adaptor proteins, Crk and CrkL, as well as other signaling components, before it is degraded by the ubiquitin-proteasome pathway. These adaptor proteins induce cytoskeletal changes and other cellular responses necessary for appropriate migration and positioning of neurons. Therefore, Dab1 plays a central role in Reelin signaling, and failure of either expression or phosphorylation of Dab1 leads to severe defects in neuronal migration, similar to those observed in the absence of Reelin or Reelin receptors. In this issue of *Neuron*, Yano et al. present a novel mechanism of Dab1 regulation involving alternative splicing by Nova2.

Nova2 is an RNA-binding protein specifically expressed in neurons. It was identified as an autoantigen in paraneoplastic opsoclonus myoclonus ataxia (POMA) (Yang et al., 1998), a neurologic

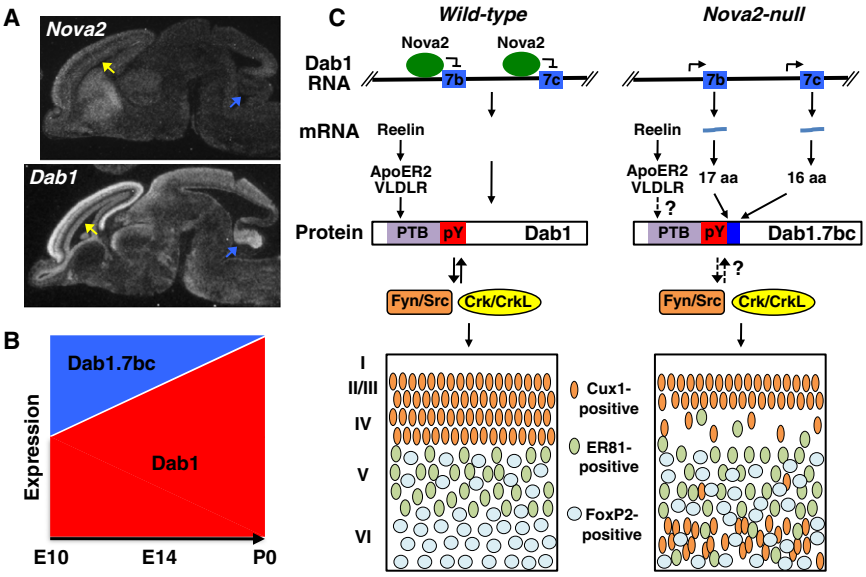

Figure 1. Alternative Splicing of Dab1 and Neuronal Migration in the Developing Brain

(A) In situ hybridization analysis of E15 mouse brain from www.chopbgem.org. Note that expression of Nova2 is weak in the ventricular and intermediate zone of the cortex (yellow arrow) and in the cerebellum (blue arrow), relative to that of Dab1.

(B) Expression of Dab1 and its splice variant Dab1.7bc in the developing mouse brain.

(C) RNA processing of Dab1 and neuronal migration in wild-type and Nova2 null mice. In wild-type mice, Nova2 binds to YCAY motifs immediately upstream of exons 7b and 7c, leading to skipping of these exons during splicing. In Nova2 null mice, exons 7b and 7c are incorporated into mRNA, leading to the addition of 33 amino acids juxtaposed to the phosphotyrosine residues. Dab1.7bc inhibits neuronal migration by unknown mechanisms.

disorder characterized by ataxia and tremors—phenotypes that are characteristic of mice with defects in the Reelin pathway. POMA results from an autoimmune attack against antigens expressed by tumor cells that are also present in normal neurons. Using antibodies from patient sera, they identified Nova2, as well as Nova1, as autoantigens responsible for disease. Nova2 is a sequence-specific RNA-binding protein that is highly expressed in neurons in the developing and postnatal brain, suggesting a unique function in regulating RNA processing in neuronal cells. To identify RNAs to which Nova binds, Darnell and colleagues purified protein-RNA complexes using ultraviolet cross-linking and immunoprecipitation (CLIP). CLIP analysis led them to identify target transcripts alternatively transcribed by Nova, as well as map the RNA binding sites involved in exon exclusion or inclusion (Ule et al., 2003, 2006).

Here, Yano et al. (2010) examine Nova2 null mouse brain to determine whether laminar structures are formed normally. They found that Cux1-positive cortical neurons, usually positioned in neuronal layers II-IV, are mislocalized underneath layer V in Nova2 null mice (Figure 1C). Early generated neurons such as ER81-positive neurons, normally destined to form layer V, also exhibited mild mislocalization in the subventricular zone. The results demonstrate that migration of late-generated neurons is severely affected in the absence of Nova2, while early-generated neurons are only mildly affected. Based on the information presented by Yano et al. (2010), it seems unlikely that the preplate fails to split into the marginal zone and the subplate as the marginal zone is not crowded in Nova2 null mice. Failure of preplate splitting and accumulation of cells in the marginal zone are hallmarks of *reeler*, Dab1 null, and other Reelin pathway mutant mice (Rice et al., 1998; Park and Curran, 2008). Therefore, Nova2 null mice may exhibit a partial neuronal migration defect distinct from that observed in Reelin pathway mutant mice. The authors also find that only 30% of Purkinje neurons failed to migrate, remaining within cerebellar white matter, in Nova2 null mice. Additional histological studies will shed light on these processes in Nova2 null mice, and it will be interesting to determine whether layer formation and dendritic development occur normally in the hippocampus, since these processes are defective in *reeler* and in mice lacking Crk and CrkL (Park and Curran, 2008).

Yano et al. (2010) also investigate the underlying mechanism whereby Nova2 contributes to neuronal migration. Nova2 is a RNA-binding protein previously shown to regulate alternative splicing. The authors utilized the approach they developed, high-throughput sequencing of RNAs isolated by CLIP (HITS-CLIP), to identify RNA targets of Nova2 in the developing mouse brain. Among 20 transcripts encoding Reelin and other proteins involved in migration, only Dab1 showed highly significant splicing changes in Nova2 null embryos. RT-PCR analysis of wild-type cortex revealed the presence of both Dab1 and its splice variant, Dab1.7bc. Dab1.7bc includes 17 amino acids from exon 7b and 16 amino acids from exon 7c in addition to the complete 555 amino acids of Dab1 (Figure 1C). Expression of Dab1.7bc is dramatically reduced at E14.5, and completely absent at E16.5 through P10, indicating that it undergoes temporal regulation during brain development (Figure 1B). In Nova2 null mice, Dab1.7bc is dramatically increased from E10 through E16.5, suggesting that Nova2 suppresses expression of Dab1.7bc during late stages of embryonic development. To assess whether Nova2 directly acts on Dab1 RNA to exclude exons 7b and 7c, Yano et al. (2010) mutated Nova2 binding *cis*-acting elements (YCAY motifs) immediate upstream of exons 7b and 7c and carried out reporter minigene assays in 293T cells. Cotransfection with Nova2 blocked inclusion of exons 7b and 7c in Dab1; however, mutation of the YCAY motifs selectively inhibited this effect. These results clearly indicate that Nova2 directly suppresses inclusion of Dab1.7bc.

To address whether neuronal migration defects in the absence of Nova2 are a consequence of increased Dab1.7bc expression, Yano et al. (2010) employed an in utero electroporation approach. Remarkably, electroporation of E14.5 cortex with Dab1.7bc caused a neuronal

migration defect similar to that seen in Nova2 null mice. Furthermore, electroporation with wild-type Dab1 overcame the neuronal migration defect in Nova2 null mice. These reciprocal experiments suggest that Dab1 and Dab1.7bc act antagonistically to regulate neuronal migration. Thus, neuronal migration defects can be attributed to either a decrease in Dab1 or an increase in Dab1.7bc. When these two conditions occur simultaneously, the defect is enhanced.

It should be noted that, unlike *reeler* mice, neuronal migration is not completely disrupted in the cerebral cortex and cerebellum in the absence of Nova2. Many Cux1-positive cells migrated normally in Nova2 null mice (Figure 1C). A comparison of the gene expression patterns of Nova2 and Dab1 in the developing brain provides a clue to explain this discrepancy. According to both immunostaining results by Yano et al. (2010) and in situ hybridization data from www.chopbgem.org (Magdaleno et al., 2006), Nova2 is expressed mainly in the cortical plate of the developing cortex. On the other hand, Dab1 is expressed in the ventricular zone as well as the cortical plate and the intermediate zone. Nova2 expression appears to be relatively lower than that of Dab1 in the developing cerebral cortex and cerebellum (Figure 1A). Therefore, it is possible that either Nova2 must reach a critical level of expression to affect a subpopulation of neurons during migration or that it influences neuronal migration independently of the Reelin pathway. In situ hybridization analysis using probes specific for exons 7b and 7c would be helpful to address this issue further.

An important question remains concerning the mechanism whereby Dab1.7bc inhibits neuronal migration. Although the authors show that Dab1.7bc can be tyrosine phosphorylated by Src causing it to form a complex with Crk in 293T cells, it is unclear whether this happens during neuronal migration in the Nova2 null embryonic cortex. In 293T cells, Dab1.7bc was not degraded upon tyrosine phosphorylation in the presence of ectopic Src, suggesting that, unlike Dab1, it might be resistant to degradation by the ubiquitin-proteasome pathway. Failure of Dab1 protein degradation and its accumulation is a good indicator of ectopic neurons, since elevated levels of Dab1 proteins were observed in displaced neurons in *reeler* (Rice et al., 1998). However, the defect in neuronal migration observed in the absence of Nova2, or in the presence of Dab1.7bc, is different from the excess migration defect observed in mice when the E3 ubiquitin ligase cullin 5 is ablated and active Dab1 accumulates in migrating neurons (Feng et al., 2007). The phenotype of Nova2 null mice is more akin to that of ApoER2 knockout mice, in which late-generated neurons fail to migrate. Therefore, it is important to determine whether the Dab1.7bc protein level is elevated in developing mouse brain.

The findings presented by Yano et al. (2010) suggest that the in-frame addition of 33 amino acids in Dab1 causes a conformation change that disrupts its function in the Reelin pathway. Because Dab1.7bc induces a defect in neuronal migration in the presence of Dab1 in the in utero electroporation assay, Dab1.7bc may act in a dominant-negative manner. This is possible if Dab1.7bc preserves its ability to bind Reelin receptors upon Reelin stimulation while losing the ability to interact with Src family tyrosine kinases and other downstream molecules such as Crk and CrkL (Figure 1C). However, we cannot rule out another possibility that Dab1.7bc gains a new function that inhibits neuronal migration independently of Reelin signaling. Structural analyses could address the conformational change in the PTB and phosphotyrosine domains of Dab1.7bc. In addition, further functional analyses may shed light on whether tyrosine residues in Dab1.7bc are phosphorylated and capable of interacting with Crk and CrkL in vivo.

The work by Yano et al. (2010) provides a telling example of how HITS-CLIP analysis can be applied to identify targets of RNA-binding proteins with biological significance. The authors establish a novel mechanism for the control of neuronal migration by alternative splicing. In addition to posttranslational modification, alternative splicing of key signaling proteins, such as Dab1, provides a fine-tuning approach to modulation of complex biological processes like neuronal migration in a temporal and spatial pattern. Similar approaches may be fruitful to investigate other complex events that contribute to the development and function of the mammalian brain.

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